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Human serum hyaluronidase: Characterization of a clinical assay

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Abstract

Hyaluronidase, a lysosomal endoglycosidase mediating hyaluronan (hyaluronic acid) turnover, is thought to be important in many normal developmental and certain pathologic processes. Previous assays of serum hyaluronidase are limited with respect to their applicability for routine clinical chemistry or clinical biochemical genetics applications. We describe a new assay of human serum or plasma hyaluronidase activity based on the determination of released N-acetylglucosamine reducing termini that allows the analysis of the enzyme with small, easily obtained sample volumes. Using 10 μl of serum or plasma, sodium formate buffer and human umbilical cord hyaluronan as substrate, we found a pH optimum of 3.9 and a K_m and V_{max} of 114 mg/l and 5102 mU/l, respectively. In addition, the assay has excellent linearity, precision and reproducibility.

Keywords: Hyaluronidase; Hyaluronan; Hyaluronic acid; Hyaluronate

1. Introduction

Hyaluronidase (EC 3.2.1.35) is a lysosomal endoglycosidase that cleaves 1β4-N-acetylglucosaminide linkages of several glycosaminoglycans: hyaluronan (hyaluronic acid), chondroitin 4-sulfate and chondroitin 6-

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sulfate [1,2]. These glycosaminoglycans are present in the extracellular matrix of tissues and have important structural roles in tissues. Interactions between cells and these molecules, including hyaluronan, are important in cell migration during embryogenesis and organogenesis and cellular differentiation [3,4]. Hyaluronidase, because of its enzymatic activities, is postulated to be important in these processes, as well as in the defense against certain infectious pathogens and some malignancies and in wound healing [5–7]. Moreover, by analogy to other lysosomal enzymes [8], a genetic deficiency of hyaluronidase must exist that could be associated with a lysosomal deficiency disorder. The development of a simple serum or plasma assay for this enzyme, therefore, will be of benefit in screening for abnormalities of hyaluronidase activity associated with different pathophysiologic states.

Hyaluronidase activity has been measured using a variety of substrates, techniques and sources [9]. The few assays that have been used for the analysis of serum hyaluronidase are labor intensive and therefore difficult to use for routine clinical purposes, incompletely characterized or validated for clinically important parameters, or require critical and non-commercially available reagents [10–17]. In this report, we describe a simple and inexpensive method for the determination of serum hyaluronidase activity that has good precision and reproducibility and that can be used in the clinical evaluation of this enzymatic activity.

2. Materials and methods

2.1. Materials

The substrate, human umbilical cord hyaluronan, and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO). Serum and plasma samples for the hyaluronidase assay were obtained from apparently healthy normal control individuals with informed consent.

2.2. Hyaluronidase assay

We assayed hyaluronidase activities in serum and plasma by incubating 10 μl of serum or plasma with 250 μl of buffered substrate solution (0.10 mol/l sodium formate, pH 3.9, containing 0.1 mol/l sodium chloride, 250 g/l hyaluronan, and 1.5 mmol/l saccharic acid 1,4-lactone) for 4 h at 37°C, unless otherwise indicated. The reaction was terminated by the addition of borate buffer and reducing N-acetylglucosamine termini were determined by the Reissig reaction [18]. Specifically, to terminate the enzyme reaction, 50 μl of 0.8 mol/L potassium tetraborate, pH 9.1, was added to each sample; the tubes were heated for 3 min in a boiling water bath and then cooled in tap water [18]. The p-dimethylaminobenzaldehyde reagent (1.5 ml), made as described [18], was added, the samples vortexed, heated at 37°C for 20 min,

briefly centrifuged and then read at 585 nm. Blanks for the reaction consisted of tubes in which the buffered substrate was incubated for 4 h at 37°C in the absence of serum or plasma and which subsequently received the borate buffer, then serum, and then treated as above. A standard curve was constructed using known concentrations of N-acetylglucosamine.

In determining the pH profile and kinetic analyses, a pool of normal human sera was used. The K_m and V_{max} were determined using a Lineweaver-Burk analysis. For all studies, a unit of hyaluronidase activity was defined as the production of a micromole of reaction product (reducing terminal N-acetyglucosamine) per min at 37°C.

3. Results and discussion

Fig. 1 illustrates the effect of pH on serum hyaluronidase activity. The enzyme has a pH optimum of pH 3.9, a shoulder of activity at pH 3.4, and

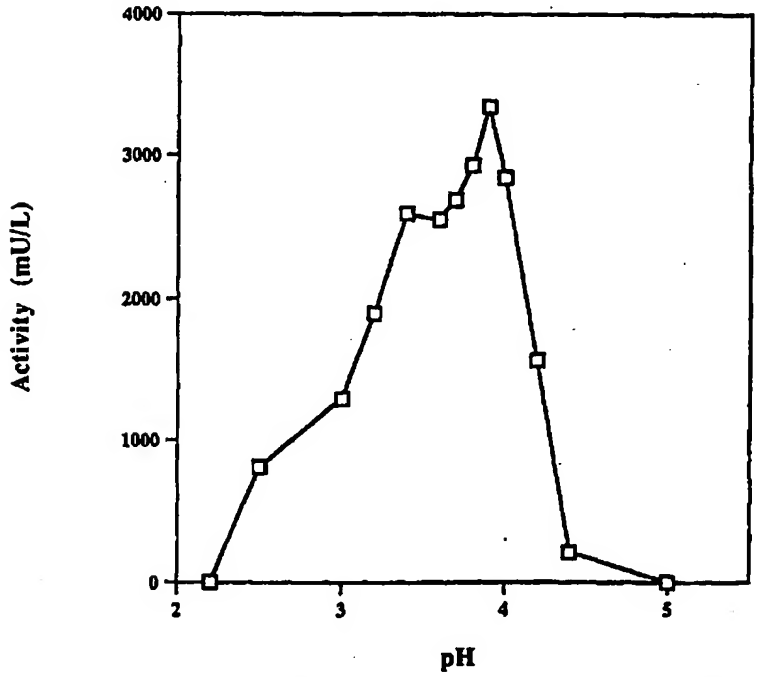

Fig. 1. pH activity profile of human serum hyaluronidase. A pool of normal human sera was assayed at the indicated pH values using a formate buffer as detailed in Methods.

no activity at pH ≥ 5.0. At pH 3.9, the serum enzyme has a K_m of 114 mg/l and a V_{max} of 5102 mU/l for human umbilical cord hyaluronan. Previous analyses of human serum hyaluronidase noted pH optima from 3.7 to 4.2 [10–14,17]. In nearly all of these studies, little or no activity was observed at pH > 5, similar to our observation. The acidic pH optimum noted here is consistent with an identity of serum hyaluronidase as a lysosomal enzyme, as has been previously noted in subcellular fractionation studies of liver, bone, spleen, submandibular gland and brain hyaluronidase [2]. There are no published data from other studies regarding the K_m of human serum hyaluronidase; the K_m values for human liver and human placental hyaluronidase against human umbilical cord hyaluronan were 50 and 118 mg/l, respectively [19,20], in good agreement with our data.

Other relevant parameters were also investigated. When using 10 μl of serum, the assay was linear up to 5 h of incubation. The assay was also linear when 3.5–15 μl serum was used in a 4 h incubation (data not shown). Intra assay variability was assessed by assaying two normal control samples, one with average activity and one with high activity, five times in the same run. The mean and standard deviation for the average activity sample were 3926 mU/l and 180 mU/l (CV 4.6%), and the mean and standard deviation for the high activity sample were 10 348 mU/l and 768 mU/l (CV 7.4%), respectively. Inter-assay variability was examined by analyzing the same control specimen on five different occasions. The mean and standard deviation in that analysis were 3541 mU/l and 296 mU/l (CV 8.4%).

We determined a normal reference range by measuring hyaluronidase activity in sera from 100 apparently healthy control individuals, 50 adult males and 50 adult females. The mean hyaluronidase activity of the control subjects was 4476 mU/l (S.D. 1144 mU/l; range 2488–9051 mU/l) (Fig. 2).

We tested for possible interferences by substances commonly found in excess in some clinical samples. Bilirubin at concentrations of 0.025 and 0.1 g/l had no effect on hyaluronidase activity; glucose at concentrations of 1 and 4 g/l had no effect but there was 30% inhibition of enzyme activity at 10 g/l (data not shown).

We also found no significant difference between values obtained for matched serum (3027 ± 710) and plasma (3280 ± 526) hyaluronidase activities. Analysis of the matched pairs yielded a confidence level of 99%, indicating that either type of specimen is appropriate for this assay.

Of the few described assays for human serum hyaluronidase activity, some are too labor intensive to be used as routine clinical diagnostic tests; all of the assays are inadequately characterized and/or validated with respect to standard clinical chemistry parameters [10–16]. One recently described assay uses an ELISA-like methodology and has excellent sensitivity but requires a key and non-commercially available reagent, a cartilage-derived high affinity hyaluronic acid binding protein [16,17]. In addition, since the substrate con-

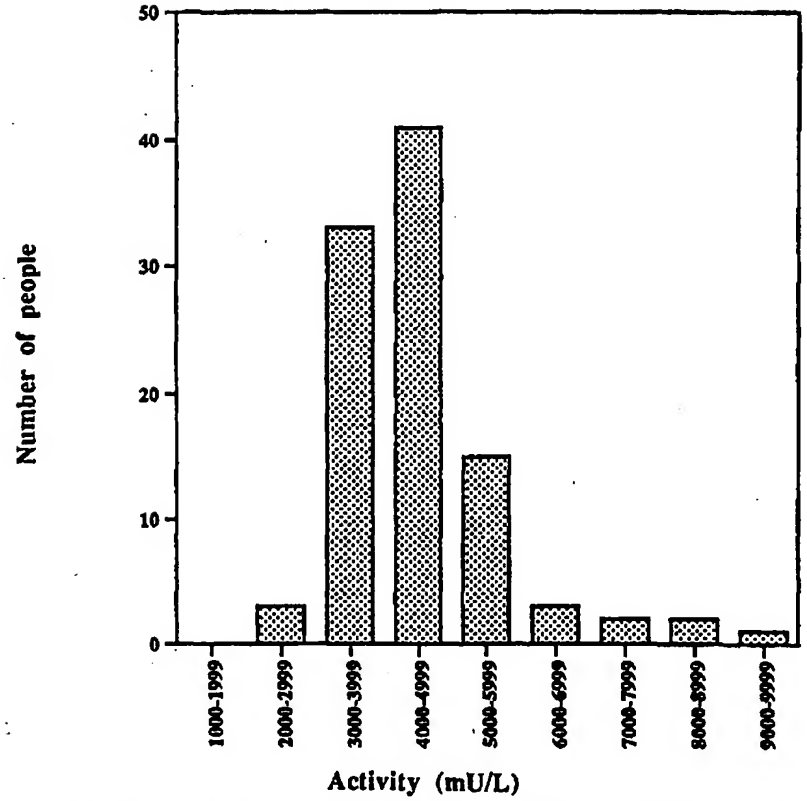

Fig. 2. Reference range of human serum hyaluronidase activity. Sera from 100 normal individuals were assayed as described in Methods.

centration in that assay can not be calculated, it is not possible to perform enzyme kinetic analyses with that method [16,17]. This limitation is highly significant since some clinically important mutations in humans are K_m or V_{max} mutations that require kinetic analyses in order to be diagnosed [see, for example, 21,22]. We should add that the clinical utility of our assay was recently verified through the discovery of a genetic deficiency of hyaluronidase activity in a child with a previously undiagnosed lysosomal storage disease [M. Natowicz et al., in preparation].

In summary, we developed a practical and inexpensive assay to determine human serum or plasma hyaluronidase activity. The assay utilizes commercially available and inexpensive reagents. The assay has excellent precision and reproducibility and excellent sensitivity for clinical applications, requiring only very small volumes of serum or plasma, and can potentially be automated.

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